PMSF (0.2 mM, EDTA (1.0 mM), 0.5% Nonidet P-40 (NP-40), and 3-[3-cholamido propyl)-dimethylammonio]-1-propane sulfonate (CHAPS; 25 mM) was added carefully, mixed for 2.5 h and centrifuged for 15 min at 10000 rpm to remove any insoluble material. Duplicate or triplicate samples were taken for protein determination [11]. Samples were stored at −80 C prior to isoelectric focusing (IEF).

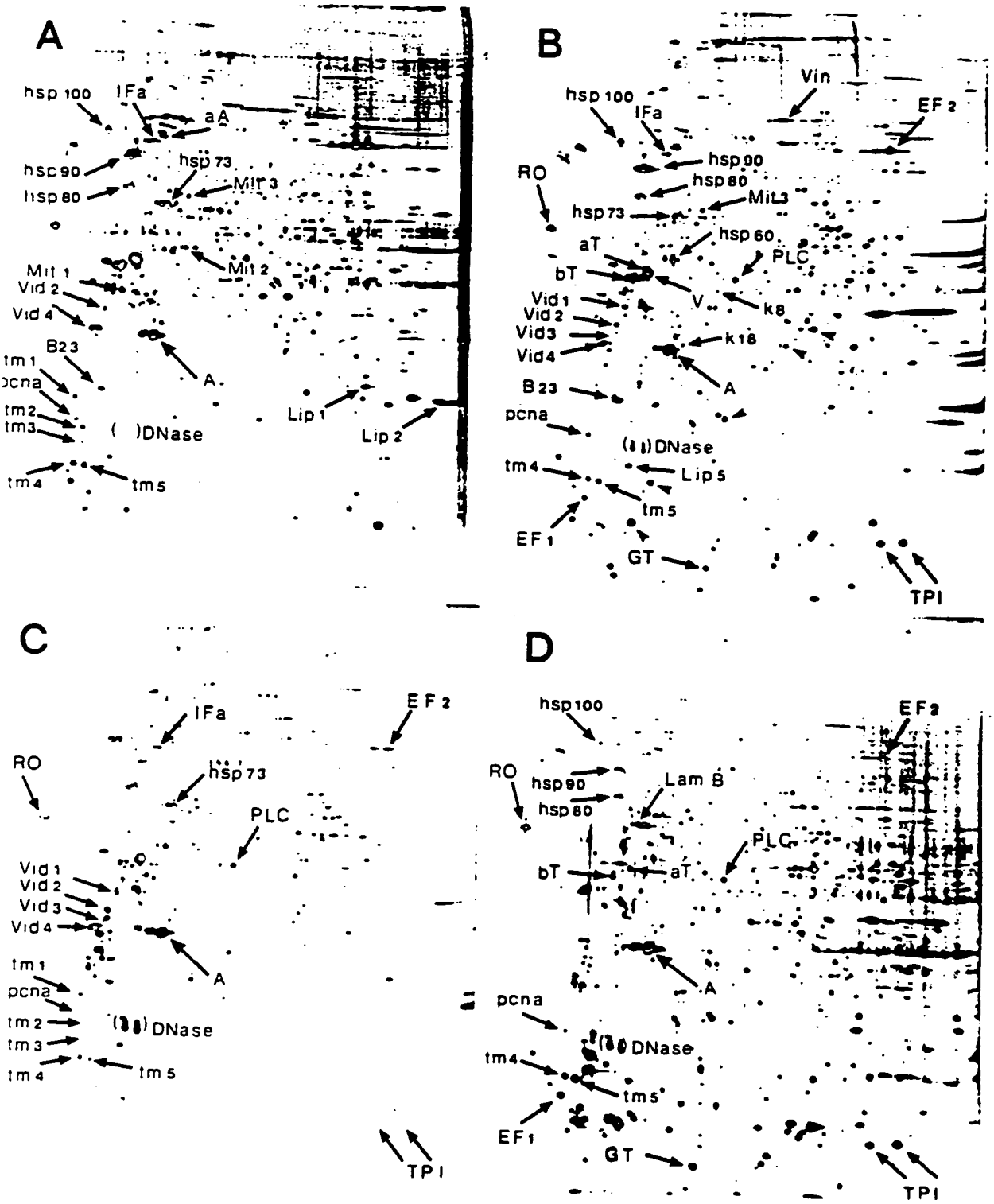

Figure 2. 2-DE analysis of samples from three cell lines and one leukemia used for the identification of polypeptides: (A) WT2; (B) MDA-231, arrowheads mark some low molecular weight cytosolic polypeptides; (C) WI38 and (D) pre B-All. The abbreviations for identified spots are explained in Table 1.

2.4.5 Preparation of frozen tumor tissue

The technique has been described previously [5,12]. Briefly, the sample is moated frozen to a fine powder, homogenized, lyophilized and solubilized in sample buffer.

2.4.6 Control of representativity

The tumors were examined routinely by experienced pathologists and smears or imprints from the samples were also assessed for cytometric DNA content by microspectrophotometry.

2.5 2-D PAGE

2-D PAGE was performed as described [8,10] except for the following details. The glass tubes for IEF, 1.2 × 200 mm, contained 2.0% Resolyte, pH 4–8 (BDH) and were cast to a height of 180 mm. A stock solution of acrylamide (Serva) and N,N'-methylenebisacrylamide (16.7:1 for IEF and 37.5:1 for the second dimension) was deionized by mixing with 5% w/v Duolite MB 5313 mixed-resin ion exchanger (BDH) for 30 min, filtered (with a 0.22 μm nitrocellulose filter) and stored at −70°C. N,N'-Methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad. IEF tubes were prefocused at 200 V in 60 min. To each tube a sample corresponding to 20–40 μg protein was applied and focused for 14.5 h at 800 V and finally 1.0 h at 1000 V using a Protean II cell (Bio-Rad) and Model 1000/500 Power Supply (Bio-Rad). The tube gels were finally extruded into 1.25 mL equilibration buffer, containing 60 mM Tris, pH 6.8 (2% SDS, 100 mM dithiothreitol and 10% glycerol), frozen on dry ice and stored at −70°C. The second dimension (1.0 × 180 × 90 mm) of the acrylamide concentration was 10% T, and the gel contained 375 mM Tris, pH 8.8, and 0.1% SDS. IEF gels were applied on top of the slab gel, sealed with 0.5% agarose containing electrophoresis running buffer (60 mM Tris-base, 0.2 M glycine and 0.1% SDS) and electrophoresed with 10–11 mA per gel (constant current) at −10°C. Six gels were run together in a Protean II xi 2-D Multi-Cell (Bio-Rad). Proteins were visualized by silver staining and photographed with the acidic side to the left [13,14].

2.6 Identification of polypeptides

Vimentin and vimentin-derived polypeptides were identified by extraction of an MDA-231 cell lysate with 0.6 M KCl/0.5% NP-40 [15]. Tropomyosins were extracted from MDA-231 and WI38 cell lysates [16], and cytokeratins were extracted from MDA-231 and MCF-7 cell lysates [17]. The patterns were compared with published maps [19–21]. Proliferating cell nuclear antigen (PCNA) was identified by immunoblotting (PC10 mAB, Dakopatt) using a semidry system (Multiphor II Nova Blot, Pharmacia-LKB Biotechnology AB) and enhanced chemoluminescence (ECL) detection (Amersham).

3 Results

3.1 2–DE of samples prepared from normal and tumorigenic cultured cells

The object of this study was to develop methods for preparation of 2-DE maps from human tumor tissue which have the same high resolution as those obtained from cultured cells. Shown in Fig. 2 are high resolution 2-DE gels prepared from cultured cells and one leukemia: SV40 transformed embryonal rat fibroblasts WT2 (Fig. 2a); human MDA-231 breast carcinoma cells (Fig. 2b); human WI38 fibroblasts (Fig. 2c) and human pre B-ALL

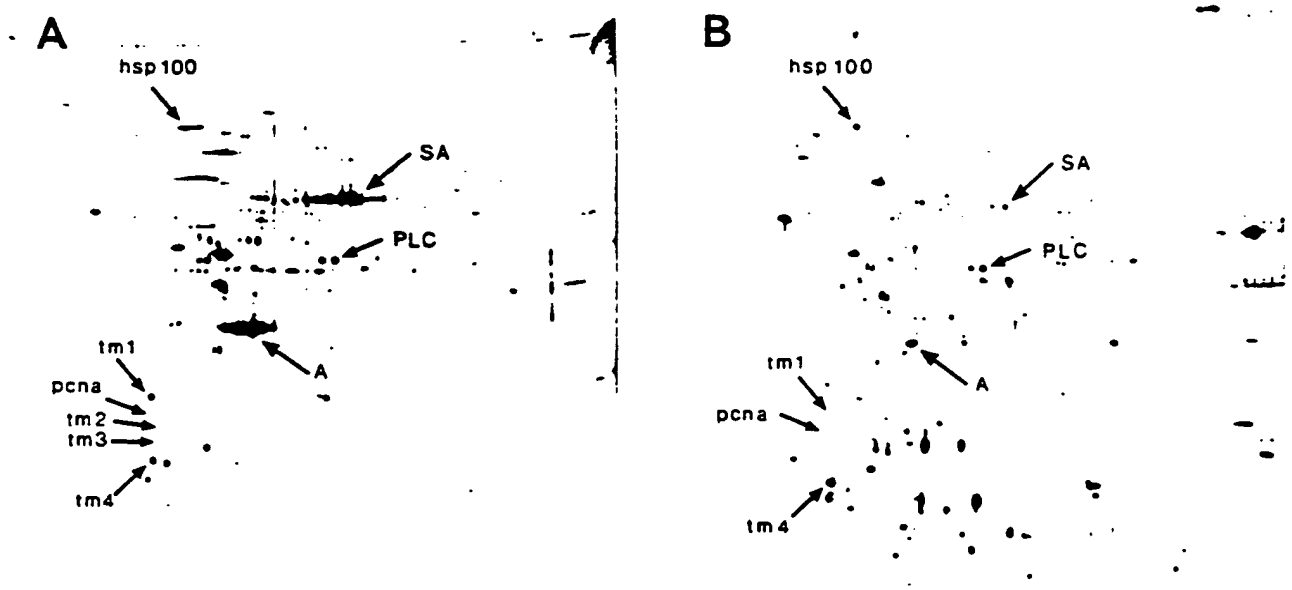

Figure 3. 2-DE analysis of a case of lung adenocarcinoma (LA). Comparison of 2-DE gel quality between (A) frozen and (B) fresh (needle aspiration) tissue preparation.

cells (Fig. 2d). Polypeptides were identified through a laboratory exchange of cell samples/2-DE maps and through 2-DE analysis of purified proteins (Table 1).

3.2 Preparation of samples from solid tumors

3.2.1 Fresh *versus* frozen tissue

An adenocarcinoma of the lung (LA) was prepared for 2-DE by conventional methods using frozen material (Fig. 3a). There are several possibilities for the poor resolution using frozen tissue, including the presence of high molecular weight protein aggregates. Filtering extracts through 0.1 μm filters (Durapore, Millipore) resulted in a slightly improved resolution (not shown). When fresh tumor tissue from tumor LA was used for sample preparation, using fine needle aspiration to collect the cells, the resolution was considerably improved (Fig. 3b). The use of fresh tissue resulted in a general increase in resolution, which was most pronounced in the 50–100 kDa molecular mass range. A number of differences in the protein profiles of the gels in Figs. 3a and 3b can be observed, some of which are indicated in the figures. The decrease in serum albumin in Fig. 3b is likely to result from loss of serum proteins occurring when cells were pelleted after aspiration. Other differences, such as the decreased level of transformation-sensitive tropomyosins (TM1-TM3), may result from enrichment of tumor cells in the sample of Fig. 3b. Fine needle aspiration, a well-established technique in cytology, extracts mainly tumor cells because of decreased intercellular adhesiveness of neoplastic cells as compared to normal tissue. Microscopic examination of Diff-Quick-stained extracted cells from case LA revealed almost 100% tumor cells, whereas the whole tissue extract contained approximately 60% tumor cells.

Table 1. Names and abbreviations for identified spots

Spot	Name	Basis for identification
A	Actins	a
aA	*alpha*-Actinin	a
B23	Protein B23 /Numatrin	a
EF2	Elongation factor 2	a
EF1	Elongation factor 1 δ	a
GT	Glutathione-S-transpherase (*pi*	a
hsp60	Heat shock protein 60	a
hsp73	Heat shock protein 73	a
hsp80	Heat shock protein 80, GRP78, BIP	a
hsp90	Heat shock protein 90	a
hsp100	Heat shock protein 100, Endoplasmin	a
IFa	Intermediary filament associated	a
k8	Cytokeratin 8	b and a
LamB	Lamin B	a
Lip1	Lipocortin I	a
Lip2	Lipocortin II	a
Lip5	Lipocortin V	a
Mit1	Mitcon 1/β – F1 ATPase	a
Mit2	Mitcon 2	a
Mit3	Mitcon 3	a
MRP	Mucine Related Polypeptides	–
pcna	Ploliferating cell nuclear antigen	c and a
PLC	Phospholipase C (1)	a
RO	RO/SS-A antigen	a
SA	Serum Albumin	b and a
aT	*alpha*-Tubulin	a
bT	*betha*-Tubulin	a
tm1	Non-muscle tropomyosin isoform 1	b and a
tm2	Non-muscle tropomyosin isoform 2	b and a
tm3	Non-muscle tropomyosin isoform 3	b and a
tm4	Non-muscle tropomyosin isoform 4	b and a
tm5	Non-muscle tropomyosin isoform 5	b and a
TPI	Triose phosphate isomerase	a
V	Vimentin	b and a
Vid1	Vimentin derived protein	b and a
Vid2	Vimentin derived protein	b and a
Vid3	Vimentin derived protein	b and a
Vid4	Vimentin derived protein	b and a
Vin	Vinculin	a

a. homologous position with respect to other mammalian systems
b. purified protein(s)
c. immunoblotting

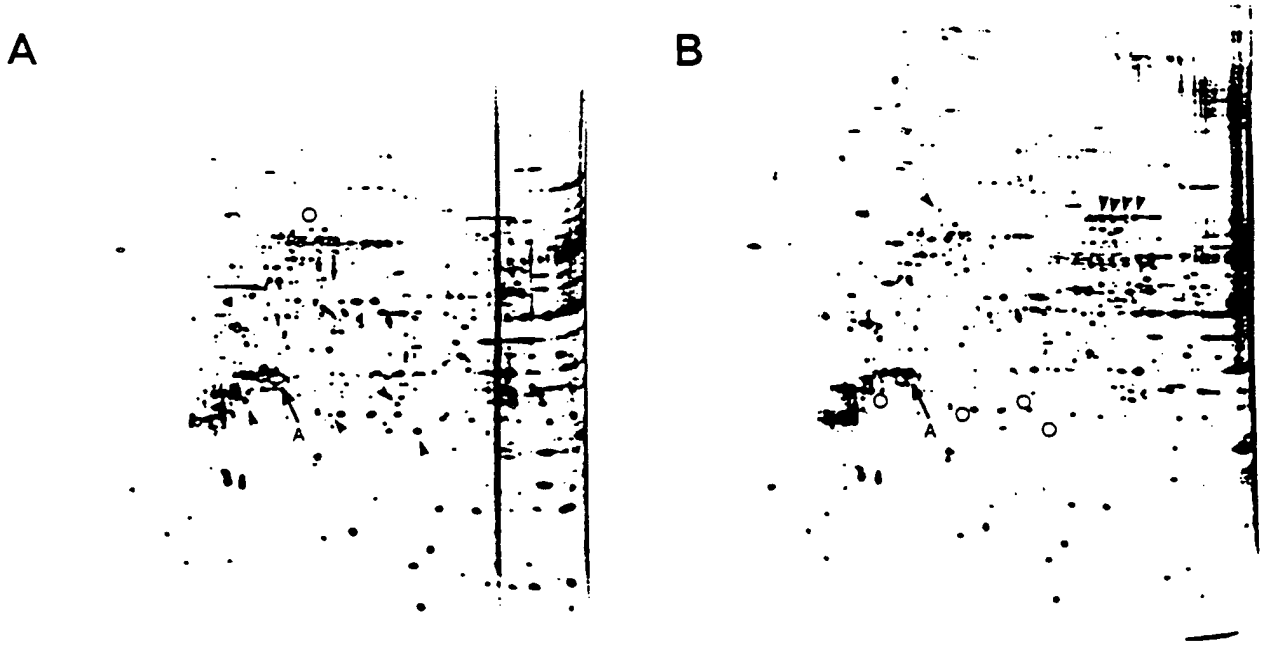

Figure 4. 2-DE analysis of a case of breast carcinoma (BC). Comparison of 2-DE quality and some differences in detected spots (arrow heads indicate increased intensity and circles or bracket indicate decreased intensity of the same spots) between (A) enzymatically and (B) nonenzymatically ("scraped") tissue preparation

3.2.2 Comparison of different methods for preparing cells from fresh tumor tissue

Samples were prepared from breast and lung carcinomas using either an enzymatic treatment with collagenase/elastase or using nonenzymatic preparations (Fig. 4). A number of differences in the protein profiles were observed in the resulting 2-DE gels, some of which are indicated in Figs. 4a and b. These differences include both increases and decreases in spot intensity. These differences may result from degradation of high molecular weight polypeptides during enzymatic treatment, increased solubilization of polypeptides, or may have other causes. For many tumors, it was only possible to obtain small amounts of material since they were reserved for other examinations. In these cases, samples could be prepared for 2-DE using either needle aspiration or scraping. Figure 5a shows a 2-DE gel prepared from squamous lung carcinoma (LS) cells collected by needle aspiration and Fig. 5b shows a gel prepared from the same tumor by scraping. In this case, a number of differences were recorded between the two procedures, some of which are arrowed in Fig. 5. Samples obtained from other tumors (breast and lung) generally showed fewer differences between these two methods of cell sampling (not shown). These data show that different nonenzymatic extraction procedures may yield different polypeptide patterns. However, the number of spots with a large

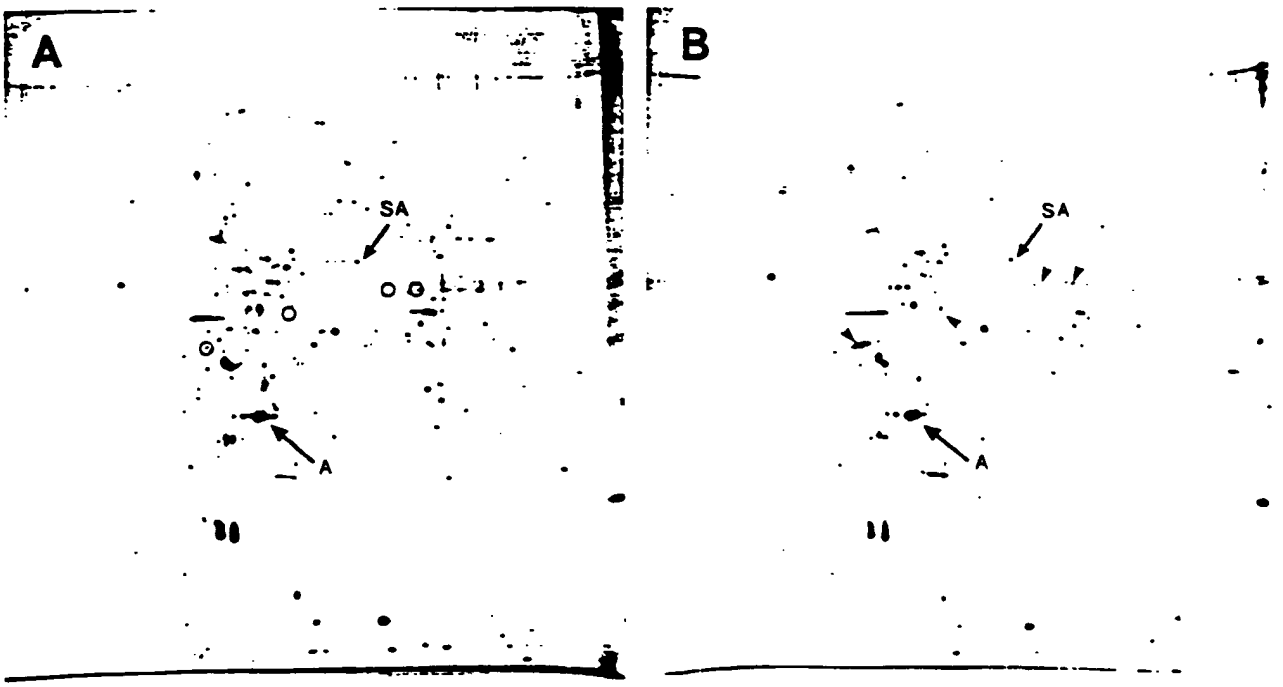

Figure 5. 2-DE analysis of a case of lung cancer (LS). Comparison of 2-DE gel quality and detected spots (arrow heads and circles) between (A) aspirated (needle aspiration) and (B) scraped preparations from fresh tissue.

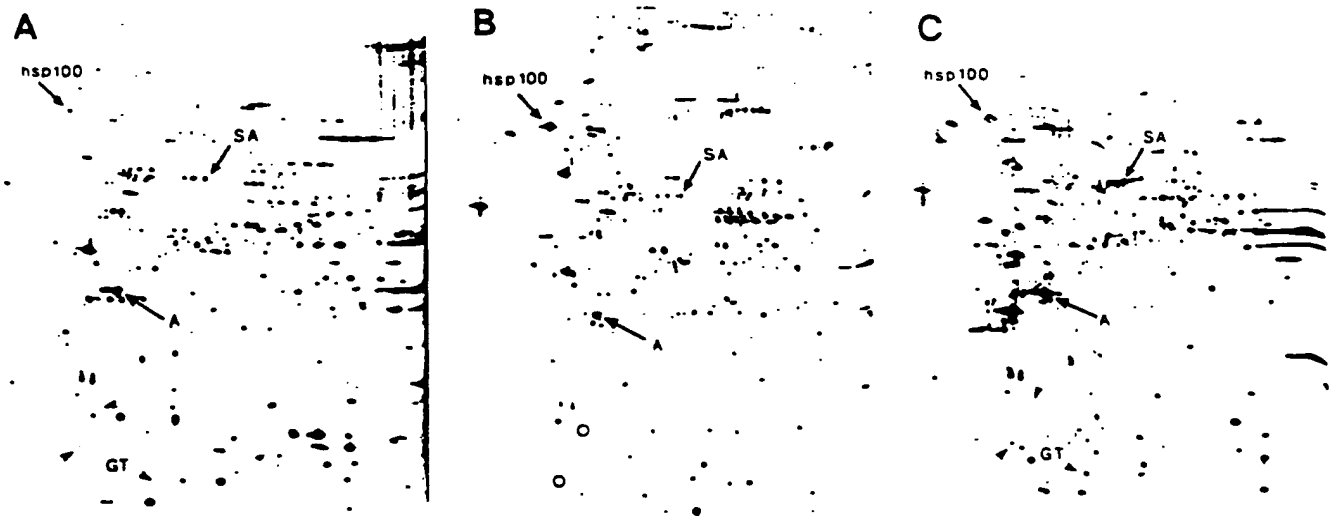

Figure 6. 2-DE analysis of three other types of tumors. (A) hypernephroma, (B) an adenoma of the thyroid and (C) corpus cancer, using the nonenzymatic preparation technique. Arrowheads and circles indicate some cytosolic polypeptides.

difference in intensity were lower than when a nonenzymatic preparation was compared with an enzymatic preparation.

2-DE maps of satisfactory quality were prepared by a third procedure. Cells were released from small pieces of tumor by squeezing (see Section 2). Some examples of this are shown in Fig. 6 where 2-DE maps derived from a case of hypernephroma, KH (Fig. 6a), a case of thyroid tumor, TA (Fig. 6b) and a case of corpus cancer, CP (Fig. 6c) can be seen. We conclude that nonenzymatic techniques are useful for 2-DE analysis of a number of different tumors. The quality of the resulting gels is comparable to that obtained using cultured cells (compare the gels in Fig. 2 with those in Fig. 4, 6 and 7). Which of these methods will be optimal will, in our experience, depend on the tumor material. For example, very small tumors are preferably extracted by squeezing; on the other hand, breast cancers (which are often fibrous) yield satisfactory samples using scraping.

3.2.3 Purification of cells on percoll gradients

We considered the possible advantage of separating viable cells from dead cells, erythrocytes, and debris using discontinuous Percoll gradients. Cells collected

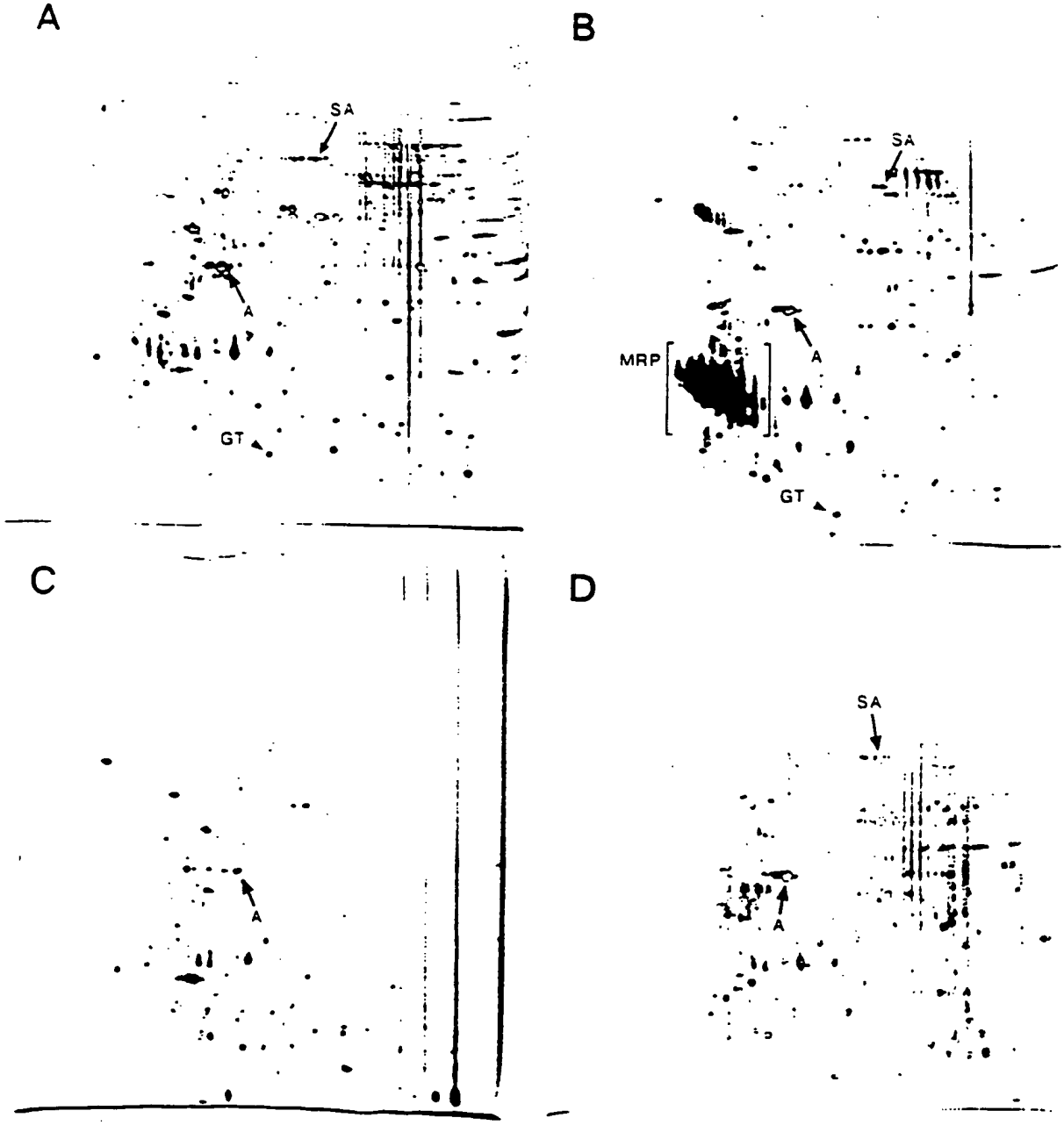

Figure 7. 2-DE analysis of polypeptides from viable (b and d) and nonviable (a and c) cells of an adenocarcinoma of the lung (LB), separated using discontinuous Percoll density gradient. Nonenzymatic preparation technique (a and b) and enzymatic preparation technique (c and d) are compared.

from the interphase showed a viability of more than 90% as judged by trypan blue exclusion test. However, it as found that the yield of viable cells decreased dramatically if the tissue resection was not immediately processed. To study the effect of lysis of cells during the preparation procedure, 2-DE maps were prepared from nonenzymatically extracted cells of case LB collected from the top fraction (nonviable, Fig. 7a) and interphase fraction (viable, Fig. 7b). These 2-DE maps were compared with corresponding fractions (nonviable, Fig. 7c, and viable, Fig. 7d) of enzymatically extracted cells. One clear disadvantage of the enzymatic technique was that when loss of cell viability occurred during preparation, a dramatic loss of high molecular weight polypeptides was observed (Fig. 7c). This was probably due to degradation of intracellular proteins. However, nonenzymatic preparations showed fewer differences between viable and nonviable cells: The most pronounced alteration was a decrease of a group of mucine related proteins (Fig. 7b). We conclude, therefore, that discontinuous Percoll gradient is necessary after enzymatic extraction of cells, but can be omitted from the nonenzymatical tumor sample preparation procedure.

We used the MDA-231 cell line to study the effects of cell lysis and leakage of cytosolic polypeptides during sample preparation. Remarkably, after 30, 50, 80 and 140 min of incubation in PBS/PIH at 0°C, no significant changes were observed in the 2-DE pattern (not shown). Although loss of cell viability may not result in protein degradation when cells are incubated in the presence of protease inhibitors, loss of cytosolic proteins would be expected during pelleting of cells. We monitored the loss of lactate dehydrogenase (LDH) activity into the supernatant during incubation in PBS of MDA-231 and MCF-7 breast cancer cells at 20°C. In both cases, loss of viability was paralleled by release of LDH from the cells (Fig. 8). After 5 h, 70% of the MCF-7 cells, but only 30% of the MDA-231 cells were dead (not shown).

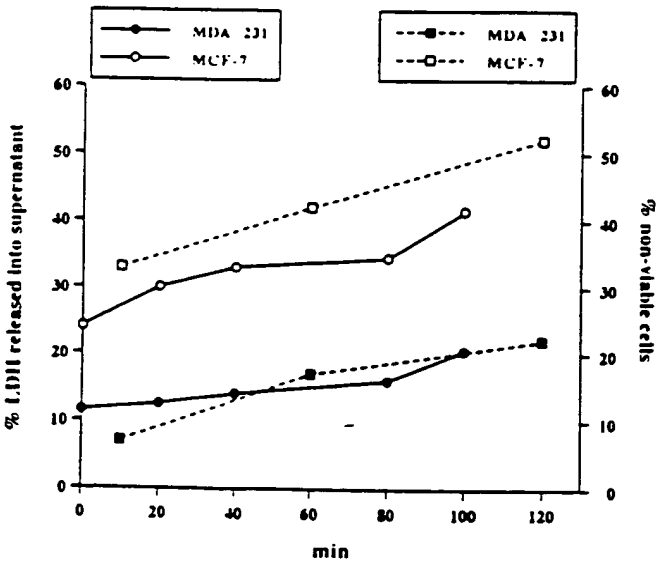

Figure 8. The relative release (fraction in supernatant of total) of lactate dehydrogenase activity (LDH) and cells viability *versus* incubation time of the mammary carcinoma cell lines MDA-231 and MCF-7 during incubation in PBS at 20°C.

These data indicate the impact of a rapid preparation procedure, at low temperature, of fresh tumor samples. Experiments have also been performed using only 1.07 g/mL Percoll (Fig. 6c and Fig. 1, left test tube) in order to remove erythrocytes. One clear advantage with this procedure, which today is routinely utilized, is a higher yield of viable cells, probably due to decreased sample preparation time.

4 Discussion

We describe procedures for sample preparation from solid tumors for 2-DE. 2-DE maps could be derived from solid tumors which were similar in quality to those obtained from cultured cells. Compared to methods using frozen material, the resolving power of the 2-DE technique is increased, allowing examination of a large number of polypeptides from tumors of different malignancies. Other investigators [12,22] have used samples from frozen tumors to derive 2-DE maps. We have previously described disadvantages encountered using frozen tumor samples including variations in contaminating proteins between different samples [3]. The methods described here are based on the preparation of cells from tumors without enzymatic digestion. The enzymatic step could be avoided since malignant cells usually grow as solid masses which are not strongly attached to the matrix. Furthermore, we found that omitting the enzymatic digestion alleviated the necessity of purifying viable tumor cells on Percoll gradients. This was in sharp contrast to enzymatically treated samples, where loss of viability leads to loss of high molecular weight proteins (Fig. 7c).

At least in the case of lung cancer, viable and nonviable cells showed small differences in respect to 2-DE maps. Presumably, protease inhibitors penetrate cells and inhibit proteolysis. In model experiments, we observed leakage of cytosolic protein (LDH) from the cells in parallel to loss of viability. Apparently, however, only a limited decrease of the level of low molecular weight cytosolic polypeptides was detected using silver staining combined with visual inspection. We have found that although some tumors are well suited for the preparation procedure described, others are not. In general, good results were obtained using tumors of the lung, breast, corpus and lymphomas. In contrast, cells from thyroid adenomas and hypernephroma showed poor viability. We were in these cases unable to separate nonviable cells from viable cells, and we can therefore not evaluate the consequence of the loss of viability on 2-DE patterns, apart from a loss of some low molecular weight cytosolic polypeptides.

Highly differentiated tumors may show lower viability as compared with poorly differentiated tumors (Dr. Farkas Vanky, personal communication). A number of samples from thyroid tumors were prepared for 2-DE but most cases showed poor viability. We believe that special care is needed during preparation of generally highly differentiated tumor groups. The difference between loss of viability/leakage of LDH of the more differentiated MCF-7 cells and the less differentiated MDA-231 cells is in line

h these observations (Fig. 8). A number of potential i interesting markers, like tropomyosin isoforms, cyto- atins and heat shock proteins, appear to be insensi- e to loss of viability during the preparation procedure. have to date made numerous observations of altera- ns in the expression of these polypeptides in breast ncers and lung cancers.

other problem that may occur, irrespective of sample paration techniques used, is admixture of lympho- es. These cases are easily detectable in smears and it y therefore be possible to select lymphocyte specific ts as "internal markers" for the 2-D PAGE analysis. dies using this approach are in progress. Many of the ypeptides identified are structural (Table 1). Since the pression of many of these polypeptides are known to y between normal and malignant cells, the possibility determine their expression simultaneously is aling. In the specific case of breast cancer, altera- ns in the expression of intermediate filament proteins tokeratins) are known to occur during tumor progres- n [23]. Other proteins known to be differentially ressed between normal cells and transformed cells tropomyosins, numatrin/B23, heat shock proteins PCNA. To this end, we have observed alterations in expression of cytokeratin 8, hsp 90, and non-muscle pomyosin isoform 2 during malignant progression. uzawa *et al.*, in preparation and Franzén *et al.*, in pre- ation).

e method of choice for sample preparation from nor tissues will depend on the properties of the tumor terial studied. It may be important to use only one thod when comparing cases within one group, as dif- ences were observed between methods. The advan- es of the nonenzymatic techniques are (i) that it mini- zes contamination with connective tissue, (ii) that blems with contamination of serum proteins are ided, and (iii) that separation of viable and dead cells not necessary. Hereby the revolving power of 2-D GE is maximized for the analysis of human tumors l studies on inter-tumor variations in gene expression facilitated. In addition, the polypeptide patterns ob- ed may be more representative for the *in vivo* tumor since the use of enzymes and incubations have been imized.

would like to thank Dr. J. I. Garrels, Dr. S. Pattersson, S. M. Hanash and Dr. J. E. Celis for making sample / 2-DE map exchanges possible. This study was supported by grants from the Swedish Cancer Society and the Cancer Society in Stockholm.

Received March 5, 1993

5 References

[1] Celis, J. E., Dejgaard, K., Madsen, P., Leffers, H., Gesser, B., Honore, B., Rasmussen, H. H., Olsen, E., Lauridsen, J. B. and Ratz, G., *Electrophoresis* 1990, *11*, 1072–1113.
[2] Garrels, J. I., Franza, B. R., Chang, C., Latter, G., *Electrophoresis* 1990, *11*, 1114–1130.
[3] Franzén, B., Iwabuchi, H., Kato, H., Lindholm, J. and Auer, G., *Electrophoresis 1991*, *12*, 509–515.
[4] Sherwood, E. R., Berg, L. A., Mitchell, N. J., McNeal, J. E., Kozlowski, J. M. and Lee, C., *J. Urology* 1990, *143*, 167–171.
[5] Endler, A. T., Young, D. S., Wold, L. E., Lieber, M. M. and Curie, R. M., *J. Clin. Chem. Clin. Biochem.* 1986, *24*, 981–992.
[6] Forchhammer, J. and Macdonald-Bravo, H., in: Celis, J. E. and Bravo, R., (eds.), *Gene Expression in Normal and Transformed Cells*, Plenum, New York 1983, pp. 291–314.
[7] Linder, S., Brzeski, H. and Ringertz, N. R., *Exp. Cell. Res.* 1979, *120*, 1–14.
[8] Celis, J. E. and Bravo, R. (Eds.), *Two-dimensional Gel Electrophoresis of Proteins*, Academic Press, New York 1984, pp. 3–36.
[9] Garrels, J. I., *J. Biol. Chem.* 1979, *254*, 7961–7977.
[10] Anderson, N. L., *Two-Dimensional Electrophoresis, Operation of the ISO-DALT System*, Large Scale Biology Press, Washington, DC 1988, 162.
[11] Bradford, M., *Anal. Biochem.* 1976, *72*, 248.
[12] Tracy, R. P., Wold, L. E., Currie, L. M. and Young, D. S., *Clin. Chem.* 1982, *28*, 890–899.
[13] Merril, C. R., Goldman, D., Sedman, S. A. and Elbert, H. M., *Science* 1981, *211*, 1437–1438.
[14] Morrissey, J. H., *Anal Biochem.* 1981, *117*, 307–310.
[15] Gard, D. L., Bell, P. B., Lazarides, E., *Proc. Natl. Acad. Sci. USA*, 1979, *76*, 3894–3898.
[16] Matsumura, F., Lin, J.-C., Yamashiko-Matsumura, S., Thomas, G. P. and Topp, W. C., *J. Biol. Chem.*, 1983, *258*, 13954–13960.
[17] Paulin, D., Forest, N. and Perreau, J., *J. Mol. Biol.* 1980, *144*, 95–101
[18] Blobel, G. A., Moll, R., Franke, W. W., Kayser, K. W. and Gould, V. E., *Am. J. Pathol.* 1985, *121*, 235–247.
[19] Ochs, D. C., McConkey, H. E. and Guard, N. L., *Exp. Cell. Res.*, 1981, *135*, 355–362.
[20] Bhattacharya, B., Gaddamanuga, L.P., Valverius, E. M., Salomon, D. S. and H. L. Cooper, *Cancer Res.* 1990, *50*, 2105–2112.
[21] Sommers, C. L., Walker-Jones, D., Heckford, S. E., Worland, P., Valverius, A., Clark, R., McCornick, F., Stampfer, M., Abularch, S. and Gelmann, E. P., *Cancer Res.* 1989, *49*, 4258–4263
[22] Trask, D. K., Band, V., Zajchwski, D. A., Yaswen, P., Suh, T. and Sager, R., *Proc. Natl. Acad. Sci. USA* 1990, *87*, 2319–2323.
[23] Trask, D. K., Bond, V., Zajchanski, D. A., Yaswen, P., Suh, T. and Sager, R., *Proc. Natl. Acad. Sci. USA* 1990, *87*, 2319–2323.

Bengt Bjellqvist*
Bodil Basse
Eydfinnur Olsen
Julio E. Celis

Institute of Medical Biochemistry and Danish Centre for Human Genome Research, Aarhus University, Aarhus

Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions

A highly reproducible, commercial and nonlinear, wide-range immobilized pH gradient (IPG) was used to generate two-dimensional (2-D) gel maps of [^{35}S]methionine-labeled proteins from noncultured, unfractionated normal human epidermal keratinocytes. Forty one proteins, common to most human cell types and recorded in the human keratinocyte 2-D gel protein database were identified in the 2-D gel maps and their isoelectric points (p*I*) were determined using narrow-range IPGs. The latter established a pH scale that allowed comparisons between 2-D gel maps generated either with other IPGs in the first dimension or with different human protein samples. Of the 41 proteins identified, a subset of 18 was defined as suitable to evaluate the correlation between calculated and experimental p*I* values for polypeptides with known composition. The variance calculated for the discrepancies between calculated and experimental p*I* values for these proteins was 0.001 pH units. Comparison of the values by the *t*-test for dependent samples (paired test) gave a *p*-level of 0.49, indicating that there is no significant difference between the calculated and experimental p*I* values. The precision of the calculated values depended on the buffer capacity of the proteins, and on average, it improved with increased buffer capacity. As shown here, the widely available information on protein sequences cannot, *a priori*, be assumed to be sufficient for calculating p*I* values because post-translational modifications, in particular *N*-terminal blockage, pose a major problem. Of the 36 proteins analyzed in this study, 18–20 were found to be *N*-terminally blocked and of these only 6 were indicated as such in databases. The probability of *N*-terminal blockage depended on the nature of the *N*-terminal group. Twenty six of the proteins had either M, S or A as *N*-terminal amino acids and of these 17–19 were blocked. Only 1 in 10 proteins containing other *N*-terminal groups were blocked.

1 Introduction

As compared with carrier ampholyte isoelectric focusing (CA-IEF), the application of immobilized pH gradients (IPGs) in the first dimension in 2-D gel electrophoresis offers improved reproducibility [1] because the nature of the pH gradient makes the resulting focusing positions insensitive to the focusing time [2] and to the type of sample applied [3]. The recently introduced ready-made IPG strips [4] seem to be an ideal substitute for the carrier ampholyte gradients, which until now have been the most commonly used first dimensions in 2-D gel electrophoresis. The availability of standardized first dimensions opens the possibility of comparing 2-D gel maps of various cell types generated in different laboratories, provided that the focusing positions of a number of easily recognizable polypeptide spots common to the cell types in question are known. Even though this approach is limited to experiments performed with the same standardized IPG, the flexibility provided by IPGs allows the pH gradient to be adjusted to the requirements of a particular experiment.

Exchange and communication of 2-D gel protein data requires a pH scale that is independent of the particular IPG used and by which the results can be described. The introduction of carbamylation trains and the relation of focusing positions to the spots in these trains represented a step forward towards solving the reproducibility problem experienced with carrier ampholyte focusing [5]. Problems associated with the use of carbamylation trains were mainly due to lack of temperature control and to the use of nonequilibrium focusing conditions. Accordingly, the pattern variation involved not only the resulting pH gradients, but also the relative spot positions as related to each other and to spots in the carbamylation trains. Even though the question of reproducibility has, to a large extent, been solved, the carbamylation trains are still not ideal as markers because the spots in the trains do not represent defined entities but rather a large number of differently carbamylated peptides having close p*I* values. As a result, the spots are large and poorly defined as compared to the ordinary polypeptide spots in 2-D gel maps.

Correspondence: Professor J. E. Celis, Institute of Medical Biochemistry and Danish Centre for Human Genome Research, Aarhus University, DK-8000 Aarhus C, Denmark

Abbreviations: CA-IEF, carrier ampholyte-isoelectric focusing; SSP, sample spot number

* Present address: Pharmacia Biotech AB, S-751 82 Uppsala, Sweden

0173-0835/94/0304-0529 $5.00+.25/0

Neidhardt *et al.* [6] defined the pH gradient in 2-D gel experiments by p*I* markers whose p*I* values were calculated from the amino acid composition. Focusing positions of other polypeptides could be predicted from their composition but the p*K* values needed for the p*I* calculations were unknown. Various groups employing this approach do not use the same p*K* values [6, 7] and therefore, the p*I* values derived in this way cannot be expected to describe the variation of the hydrogen ion activity. In spite of this fact, it is still possible to make approximate predictions of focusing positions because the p*K* values used to define the pH gradient are also used to calculate p*I* values and to predict the focusing positions. Errors in p*K* assignments are therefore compensated. A pH scale which correctly reflects the variation in hydrogen ion activity during focusing should improve the precision of the predictions, but this has never been implemented with CA-IEF focusing as a first dimension in 2-D gel electrophoresis. The main reason for this are the problems associated with pH measurements in focused gels containing high concentrations of urea.

IPGs can be described from the concentration variation of the immobilized groups, provided that the p*K* values of these groups are known for the conditions prevailing during focusing. To avoid measurements on gels, Gianazza *et al.* [8] suggested the use of p*K* values derived by addition of determined p*K* shifts. Recently, direct determinations of p*K* differences between immobilized groups in IPGs were made by determining p*I*-p*K* values in overlapping narrow-range IPGs [9, 10] and the results verified the applicability of the Gianazza approach. A description of the focusing results in a pH scale, which correctly describes the variation of the hydrogen ion activity for the focusing conditions used, not only allows the comparison of 2-D gel maps generated with different IPGs, but also opens the possibility for correlating the focusing position of a polypeptide with its composition [9]. Experiments by Bjellqvist *et al.* [9, 10] have implied that pH scales showing good correlation between calculated and experimental p*I* values can be derived for any of the conditions commonly used for focusing in connection with 2-D gel electrophoresis. These pH scales are then defined through the p*K* values of the immobilized groups in the IPG containing gel. To be useful for interlaboratory comparisons, however, the pH scale has to be defined through p*I* values of easily recognizable spots present in the 2-D gel map. So far, p*I* determinations in a useful pH scale, combined with determinations of pK values needed for p*I* calculations, have only been made for the pH range 4.5–6.5 at 10°C [9]. CA-IEF focusing as described by O'Farrell [11] does not control the temperature of the first dimension, which can be expected to be slightly above room temperature. With IPGs, the temperature commonly used is about 20°C [4, 12] or 25°C [13] and this is a critical parameter that needs to be controlled [14].

The present work was designed to compare 2-D gel maps of different cell types in a laboratory applying both CA-IEF and IPG focusing at a common temperature. To this end we have generated 2-D gel maps of proteins from noncultured, unfractionated normal human epidermal keratinocytes with IPG in the first dimension and a focusing temperature of 25°C. We have used commercial nonlinear, wide-range IPG strips which give 2-D gel maps that are closely similar to the ones resulting with the CA-IEF technique used to establish the human keratinocyte database [15]. As an initial step towards interlaboratory comparisons of results obtained with the nonlinear gradient as a first dimension we report here on the focusing positions of 41 known proteins that are common to most human cell types. The pH range covered corresponds to the range in classical CA-IEF 2-D gel electrophoresis and in order to use these proteins as internal standards for comparing 2-D gel maps generated with other IPGs we determined their p*I* values with narrow-range IPGs in the first dimension. We have compared the calculated *versus* experimental p*I* values and show that it is necessary to have further information (absence or presence and nature of posttranslational modifications), in addition to amino acid composition to be able to calculate p*I* values that correspond to the actual experimental values. The p*K* values used for the calculations are provided and the usefulness of p*I* prediction in relation to database information is discussed. Furthermore, we comment on the possibility of using experimentally determined p*I* values to verify the available database information on polypeptide composition.

2 Materials and methods

2.1 Apparatus and chemicals

Equipment for isoelectric focusing and horizontal SDS electrophoresis (Multiphor® II electrophoresis chamber, Immobiline® strip tray, Multidrive XL programmable power supply, Macrodrive power supply and Multitemp® II) was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Vertical second-dimensional gels were run in the home-made equipment described in [15]. The IPG strips with the wide-range nonlinear pH gradient were either Immobiline DryStrip® pH 3–10 NL, 180 mm or alternatively 160 mm long IPG strips with a corresponding pH gradient. In both cases the IPG strips were delivered by Pharmacia LKB. Immobiline, Pharmalyte, Ampholine, GelBond as well as PAG film and the ready-made horizontal SDS gels (ExcelGel® XL SDS 12–14) were also from Pharmacia LKB. Purified proteins and peptides were from Sigma (St. Louis, MO).

2.2 Sample preparation

Preparation and labeling of unfractionated keratinocytes as well as fibroblasts have been described in [16]. Cells were lysed in a solution containing 9.8 M urea, 2% w/v NP-40, 100 mM DTT and 2% v/v Ampholine pH 7–9.

2.3 2-D gel electrophoresis

First-dimensional focusing was performed according to Görg *et al.* [2] with some minor modifications, as described in [9]. Rehydration of the IPG strips was made in a solution containing 9.8 M urea, 2% w/v CHAPS, 10 mM DTT and 2% v/v carrier ampholyte mixture. The carrier ampholyte mixture consisted of 2 parts Pharmalyte

4–6.5, 1 part Ampholine pH 6–8 and 1 part Pharmalyte pH 8–10.5. Usually, cathodic sample application was used and the samples were diluted 2–20 times in a solution containing 9.8 M urea, 4% w/v CHAPS, 1% w/v DTT and 35 mM Tris base. For acidic application, the Tris-base was substituted with 100 mM acetic acid. The degree of dilution and sample volume (20–100 µL) depended on the particular sample and the IPG, and whether visualization of the proteins was to be done by Coomassie Brilliant Blue or silver staining. With the wide-range non-linear IPG, 10–30 µg of total protein was loaded for silver staining and 100–200 µg for Coomassie staining. Focusing was done overnight with Vh products in the range of 45–60 kVh with 160 mm long strips and 50–70 kVh with 180 mm long strips. Solubilization of polypeptides and blocking of -SH groups prior to the second-dimensional run, as well as loading on the second-dimensional gel was done as described in [9]. The stacking gel was omitted and 5–10 mm were left at the top of the second-dimensional gel for applying the IPG strip. The space was filled with electrode buffer containing 0.5% w/v agarose. Casting, running, staining and autoradiography were carried out as described in [15].

2.4 Experimental determination of p*I* values

The determination of the pK' differences between Immobilines pK' 4.6, pK' 6.2 and pK' 7.0 necessary for the calibration of the pH scale at 25°C in 9.8 M urea was done as described in [9] with the same narrow-range IPGs. The pH scale was defined by setting the pK' value of Immobiline pK' 4.6 equal to 4.61 [9] and the determined pK' differences gave the pK' values of Immobilines pK' 6.2 and pK' 7.0, equal to 5.73 and 6.54, respectively. The pK' differences found are in good agreement with values derived from [17] and [8] by extrapolation to 9.8 M urea concentration. As in [9], additional narrow-range recipes have been used for determining p*I* values. With narrow-range IPGs extending to pH values higher than the pK' value of Immobiline pK' 7.0, anodic sample application was used with acetic acid added to the sample solution. Otherwise, cathodic sample application was used with the same sample buffer as for wide-range IPGs.

2.5 Protein compositions used for p*I* calculations

With the exception of vimentin, protein compositions are from the Swiss-Prot database [18]. For vimentin, we used the data from [19], where the amino acid at position 41 is a D instead of a S. Information in the Swiss-Prot database on phosphorylation has been disregarded because it was known from earlier studies (J. E. Celis, unpublished results) that the spots in question corresponded to the unphosphorylated forms of the peptides.

2.6 Calculation of p*I* values

For the p*I* calculations it was assumed that the same pK' value could be used for an amino acid residue in all polypeptides and in all positions in the peptide except for *N*- or *C*-terminally placed amino acids. For the pK' values of the *N*-terminal amino groups the effect of the different substituents on the α-carbon were taken into account. The calculations of p*I* values were made with the aid of the IPG-maker program [20].

2.7 pK' values used for p*I* calculations

For the carboxyl terminal group and internal glutamyl and aspartyl residues the same pK' values were used as in [9]. For *C*-terminal glutamyl and aspartyl residues, separate pK' values were derived with the aid of the Taft equations [9, 21]. The pK' values of histidyl groups were calculated from the p*I* values of human carbonic anhydrase I as in [9]. For *N*-terminal glycine a pK' value of 7.50 was used. The pK' shift caused by a substituent on the α-carbon was assumed to be identical with the pK' shift the substituent caused for the amino group in the amino acid, *i.e.* 2.28 pH units were subtracted from the pK' values for the amino groups in the amino acids given in [22, 23]. The approximate pK' value of 9 for the cystenyl group was taken from [24]. For tyrosyl and arginyl groups we used the pK' values for the amino acids [22, 23]. For lysyl groups the effect of high urea concentration on amino groups was taken into account and 0.5 pH units were subtracted from the amino acid pK' value. These last three pK' values are far from the pH range under study and the results found would have been the same if lysyl and arginyl groups were assumed to be fully ionized while the ionization of tyrosyl groups were neglected. A complete list of the pK' values used is given in Table 1.

Table 1. pK' Values used for the ionizable groups in peptides 9.8 M urea, 25°C

Ionizable group	pK'
C-terminal	3.55
N-terminal	
Ala	7.59
Met	7.00
Ser	6.93
Pro	8.36
Thr	6.82
Val	7.44
Glu	7.70
Internal	
Asp	4.05
Glu	4.45
His	5.98
Cys	9
Tyr	10
Lys	10
Arg	12
C-terminal side chain groups	
Asp	4.55
Glu	4.75

2.8 Statistical analysis

Statistical comparisons of the experimental and calculated p*I* values were done on an Apple Macintosh IIsi using the statistical package Statistica/Mac, release 3.0b (from StatSoft Inc., Tulsa, Oklahoma). Calculated and experimental p*I* values were compared by the *t*-test for